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ANATOMICAL ASPECTS OF GROWTH PROLIFERATION IN *NICOTIANA TABACUM* TISSUE CULTURED *IN VITRO*[1]

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ABSTRACT

Aspects of the anatomy of tobacco stem explants and subcultured callus, growing on a nutrient medium to which varying ratios of auxin and cytokinin were added, were studied. Callus formed during explant growth generally originated from the xylem ray parenchyma, either centrifugal or centripetal to the parent xylem. Internal phloem bundles, as well as phloem parenchyma and cambial derivatives, if present, also gave rise to new growth. The pith rarely produced callus. Roots, diarch, triarch or polyarch, often di- or polystelic, produced root hairs which grew in between callus cells. Buds developed large, multicellular trichomes at the bases of which stomata with photosynthetic guard cells were frequently observed. High auxin appeared to stimulate tracheid formation and high cytokinin the density of the developing xylem.

UITTREKSEL

ANATOMIESE ASPEKTE VAN DIE GROEIVERMEERDERING IN *NICOTIANA TABACUM* WEEFSEL *IN VITRO* GEKWEEK. Aspekte van die anatomie van tabak stingel-eksplante en gesubkultiveerde kallus wat op 'n voedingsmedium onder verskillende auksien en sitokinien regimes gekweek is, is bestudeer. Kallus wat op die eksplante ontwikkel blyk hoofsaaklik uit die vaatstrale van die xileem te ontspring, hoewel interne floëembondels, en floëemparenchiem en kambiale derivate, indien aanwesig, ook tot kallusproduksie aanleiding kon gee. Die murgparenchiem het slegs by uitsondering kallusweefsel geproduseer. Wortels, twee-, drie- en meerstralig, dikwels met twee of meer vaatstele, is opgemerk, met wortelhare wat tussen die kallusparenchiem deurgroei. Knoppe met groot, multisellulêre hare op die basisse waarvan huidmondjies met fotosintetiserende sluitselle dikwels voorgekom het, is algemeen opgemerk. Hoë auksientoedienings het die aantal trageiede gestimuleer terwyl hoë sitokinientoedienings die digtheid van die xileemmasse beïnvloed het.

INTRODUCTION

This study and those reported on in two subsequent papers were concerned primarily with the sequence of events by which certain morphological characteristics develop in tobacco callus in response to varying ratios of the plant growth hormones, auxin and cytokinin. The main objective was to gain a basic understanding of the anatomy of structural development in tobacco

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tissue cultured *in vitro*, in the hope that this would serve as a basis for further investigations—especially of histochemical, microautoradiographical and ultrastructural aspects—of growth and development of cultured tissues in general.

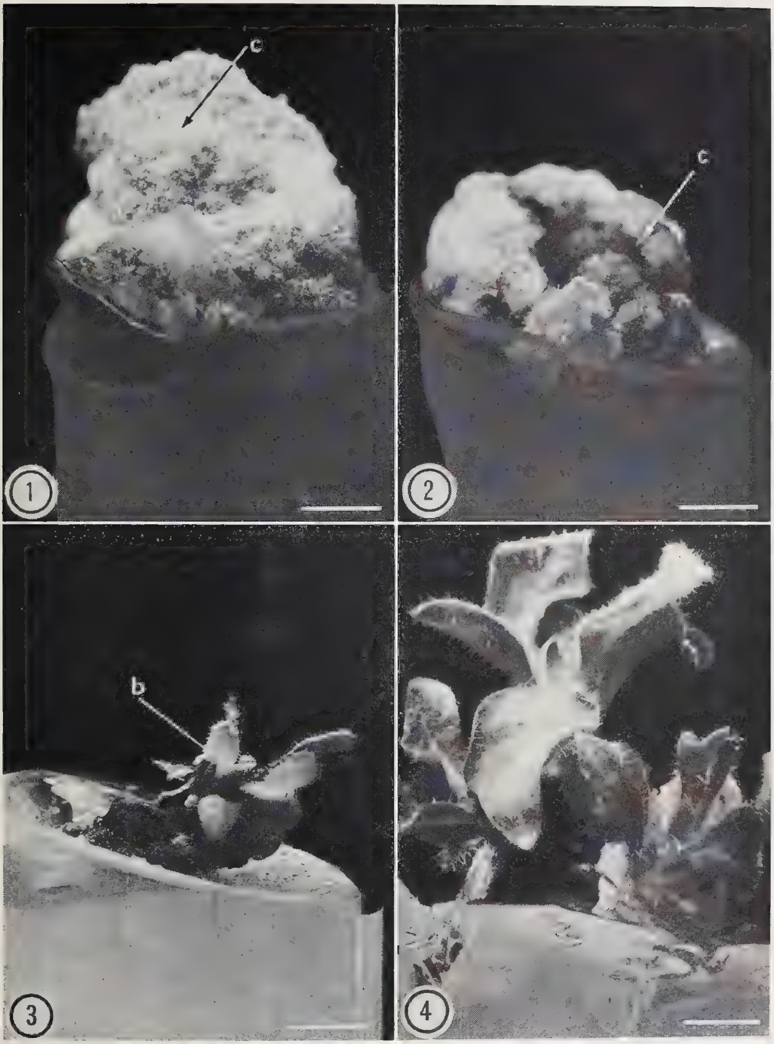
The present biochemical and physiological bias of plant tissue culture research has resulted in at least a partial neglect of basic anatomical and histogenic considerations; and, while it is generally conceded that tissue obtained by the *in vitro* subculturing of plant material is, because of its greater structural simplicity, easier to work with than the intact plant itself, this poses a fundamental problem. Karstens (1965) namely questions whether data obtained from the investigation of cultured tissues can be compared with phenomenological situations in the intact plant. Skoog (1957) maintains that plant growth and differentiation of all types is determined by quantitative levels and proportions of various growth factors, and that it would be meaningless to describe anatomical and morphological features of cultured tissues as if certain forms of growth are fixed without detailed reference to the precise conditions of growth. However, it is clear that the contributory factors to certain forms of growth can be viewed in perspective only if the basic anatomy of the original as well as the developing plant tissue is known.

In vitro culture of excised organs, tissues, and cells of higher plants has opened new approaches for studying plant cell and tissue physiology, particularly the biochemistry of morphogenesis and its control. Anatomical and physiological features of growth and development in cultured tissues are complementary and it would appear entirely fruitless to draw conclusions on the effect of, say, a particular growth substance on differentiation without relating this to, for example, the anatomical simplicity or complexity of the starting material.

For valid comparisons to be drawn between intact and cultured plants it is therefore essential that the basic structures and processes of cultured tissue be fully understood.

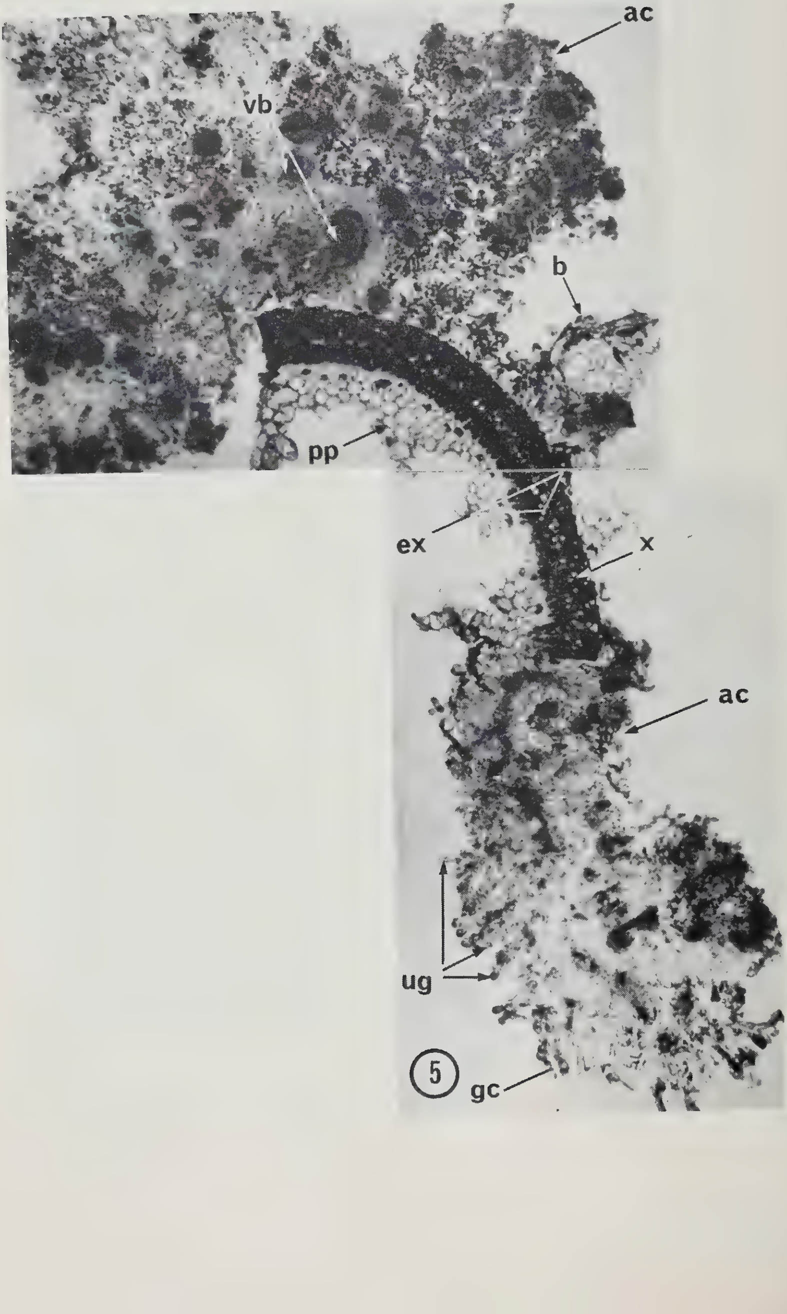
Material and methods

Explant Technique. Defoliated stems of succulent, vigorously growing *Nicotiana tabacum* (cv. Kutsaga 614) plants were sterilised by first plunging them into 70% ethanol. After thorough scrubbing, the stems were soaked in 0·35% sodium hypochlorite for 15 minutes, followed by three rinses with sterile distilled water. Four-centimetre segments were then cut and placed in sterile petri dishes. Prior to transferring to nutrient agar, one-half a centimetre of tissue was trimmed off either end and the remaining segment divided into three 1 cm pieces. The bark was then peeled away and the segments were either



FIGS. 1–4.

Tobacco callus and shoots growing on media containing different ratios of indoleacetic acid (IAA) and kinetin (CK), mg/1, respectively. Fig. 1. Loose and frosty to even and compact callus; 4·0:1·2. Fig. 2. Compact, irregularly lobed callus which did not give rise to roots but from which buds subsequently developed; 4·0:0·08. Fig. 3. Vegetative bud developing from explant on which little callus has formed; zero: 0·08. Fig. 4. Numerous buds and leafy shoots developing from explant following considerable callus formation; 4·0:0·08. Bar represents 1 cm.
b, bud; c, callus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halved or quartered. Each tissue piece was finally plunged into 70% ethanol followed by a rinse in sterile distilled water before being seated on the agar. All excisions and transfers were carried out in sterile transfer rooms.

Culture Media and Conditions. Synthetic growth media were prepared following Vasil and Hildebrandt's (1966) modification of Murashige and Skoog's (1962) medium for tobacco tissue. Auxin solutions were made by dissolving the appropriate amount of indoleacetic acid (IAA) in a few drops of 70% ethanol before diluting to volume with glass-distilled water. Kinetin (6-furfurylaminopurine) solutions were prepared by first steaming in a small volume of distilled water for about 20 minutes in an autoclave. After cooling the solutions were made to volume with glass-distilled water.

Vitamin and hormone stock solutions were prepared fresh and IAA was always made up immediately prior to use. To prevent the possible photo-inactivation of IAA by light, all final nutrient media were kept in the dark before, and for a period of three days after, transfer of the tissue. Tobacco stem explants and callus cylinders for subculture, were grown on 50 ml nutrient agar in 250 ml wide-mouth erlenmeyer flasks or on 15 ml agar in test tubes under a 12-hour photoperiod of ca. 150 ft. candles at 23 ± 3°C. The pH of the media varied from 5·5 to 5·8.

Hormone Ratios. Six different treatments were used in which the ratio of auxin to cytokinin (CK) was varied as follows: IAA : CK mg/l (1) 4·0 : zero, (2) zero : 0·08, (3) 4·0 : 0·08, (4) 4·0 : 1·2, (5) zero : 5·0, and (6) 4·0 : 5·0.

Microtechnique. A considerable amount of work was based on fresh tissue sectioned either by hand or with a sliding microtome and stained with either toluidene blue, aniline blue or a polychrome dye. Permanent slides were prepared of callus fixed in 3% glutaraldehyde or 10% acrolein, dehydrated, infiltrated and embedded following Feder and O'Brien (1968). Stains used were safranin and Delafield's haematoxylin, and Fleming's triple stain (Johansen, 1940), and aniline blue (Jensen, 1962).

Gross Morphology of the Explant. The tobacco tissue from which the explants were taken was already undergoing secondary thickening. All tissues external to the xylem except probably some elements of the vascular cambium were removed when the bark was peeled off. The remaining explant consisted of a continuous band of xylem of regular, radial rows of vessels separated by

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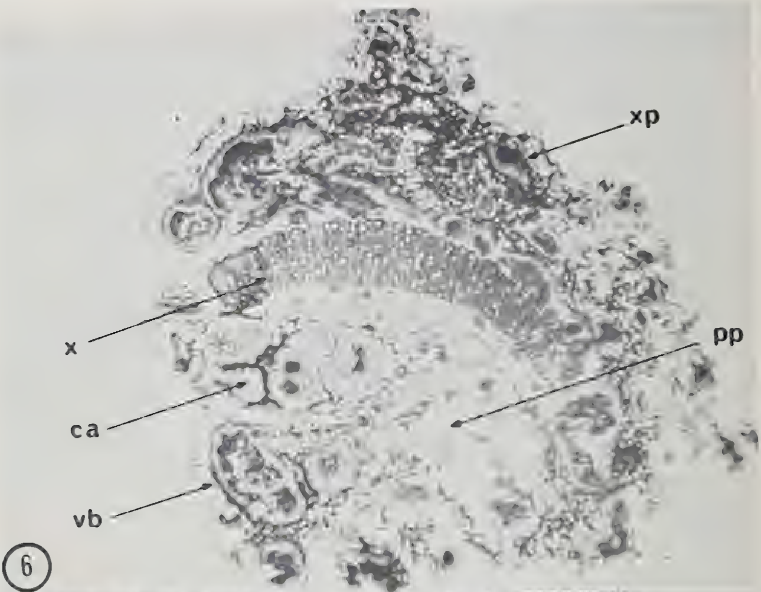
Fig. 5.

Transverse section of an explant showing callus developing from the external xylem surface as well as from peripheral internal phloem bundles. Note difference in texture between aerial callus and agar or underside callus. IAA:CK, 4·0:1·2 mg/l. Sliding microtome section of fresh tissue, polychrome stain. × 13.

ac, aerial callus; b, bud; ex, width of original explant; gc, giant cell; pp, pith parenchyma; ug, agar or underside callus, vb, vascular bundle; x, xylem.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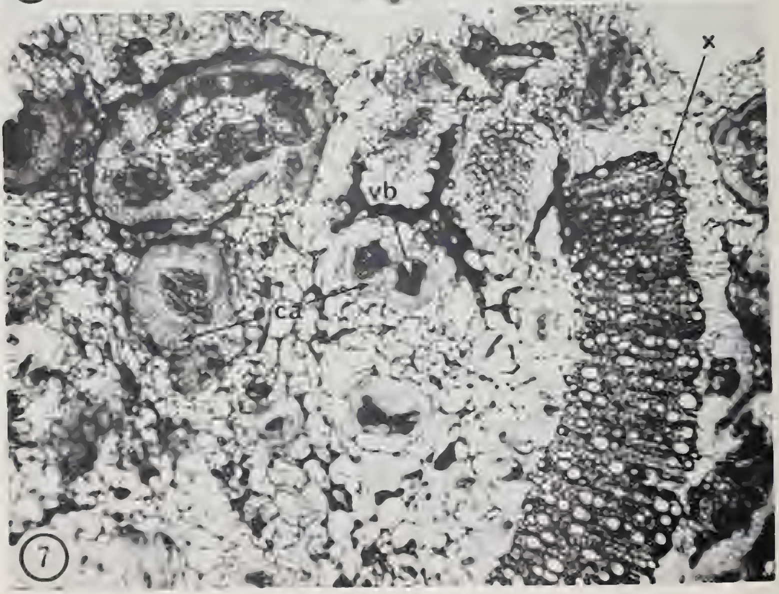
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rays of xylem parenchyma which, in older tissue, often possessed thickened walls. This band of xylem partially enclosed a pith consisting of storage parenchyma with regular, large intercellular spaces. Immediately centripetal to the xylem are groups of protoxylem elements as well as scattered bundles of internal phloem. These bundles have sieve tubes, companion cells, and phloem parenchyma as well as a lining of fibres on their inner sides. No continuous cambium is associated with the inner phloem.

RESULTS AND DISCUSSION

Despite some inconsistencies, due perhaps to the heterogeneity of the initial plant material, the six different treatments in which auxin and cytokinin ratios were varied, most commonly elicited the following growth forms:

1. IAA : CK, 4·0 : zero mg/1. Callus development was rapid and chunky in appearance with a loose, frosty surface of large, extended cells overlying a light green zone of chloroplast-containing cells. Roots, often numerous and much branched, formed from original explants following vigorous callus growth.

2. Zero : 0·08 mg/1. As expected, negligible amounts of callus were produced; not infrequently vegetative buds would develop directly from explant surface.

3. 4·0 : 0·08 mg/1. A light-green, continuous callus formed on the external xylem surface. The callus was dense and frosty but by and large remained smooth; there was no pustular growth and normally numerous buds developed.

4. 4·0 : 1·2 mg/1. An even, compact layer of green-coloured callus covering the entire surface was produced. No shoots or roots were produced.

5. Zero : 5·0 mg/1. Negligible callus growth but numerous buds were observed in the tissues internal to the explant xylem.

6. 4·0 : 5·0 mg/1. Much callus developed, appearing crystalline, frosty and lumpy. This pale frosty callus of cells overlay a deep-green tissue.

Explants with a simple and uniform anatomical structure such as tissue pieces of pure parenchyma, are probably the most suitable for histogenic studies. Those with a complex anatomy are less suitable because cells of many different

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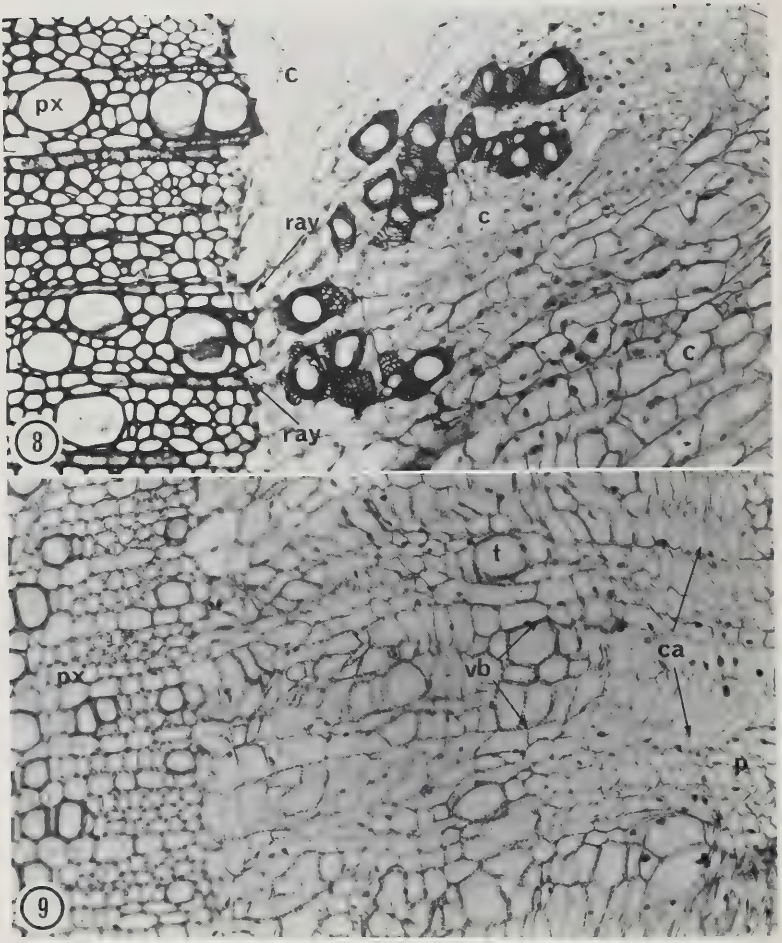
FIGS. 6–7.

Sliding microtome sections of fresh material, polychrome stain. IAA:CK, 4·0:0·08 mg/1. Fig. 6. Transverse section of cultured stem segment showing xylem and rare instance of pith proliferation. Callus is compact, with numerous vascular bundles. × 12. Fig. 7. Higher magnification of Fig. 6. showing vascular bundles surrounded by cyclic cambial layers. × 25. ca, cambial layers; pp, pith parenchyma; vb, vascular bundles; x, xylem; xp, xylem proliferations.



Figs. 8–9.

IAA:CK, 4·0:0·08 mg/l. ×160. Fig. 8. Transverse section of explant secondary xylem showing callus proliferation from xylem parenchyma of xylem ray, Fleming's triple stain. Fig. 9. In the proliferating callus a cambium with regular files of cells has formed and has produced tracheids and vascular nodules proximally and phloem distally, haematoxylin-safranin stain.

c, callus; ca. cambium; p, phloem; px, parent xylem; t, tracheid; vb, vascular bundle/nodule.

types, in various stages of differentiation and dedifferentiation, may contribute to callus proliferation, reducing the homogeneity of the subsequent tissue. The stem explants used in this study and in much of tobacco tissue culture research fall in the complex category and a great amount of variation, in fact, was encountered in the initial cultures. However, with subsequent subculturing growth forms became more uniform.

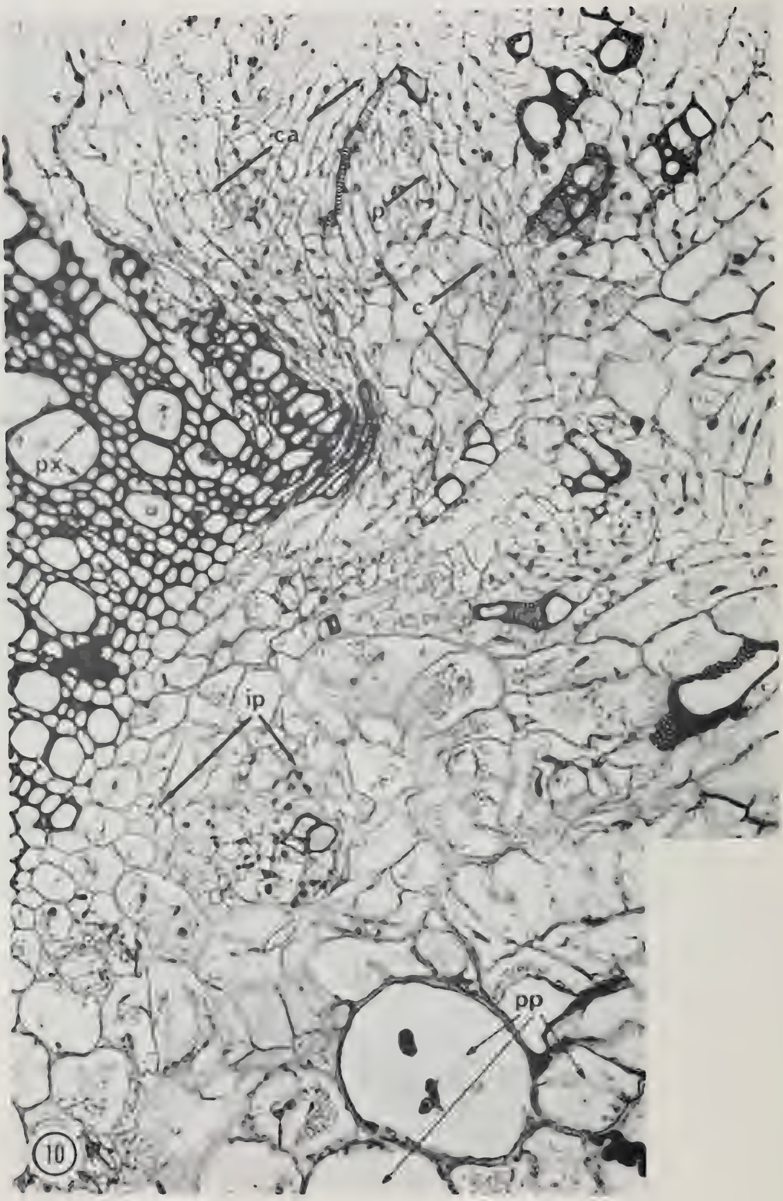
Figures 1–4 show some of the responses of tobacco stem explants to different ratios of auxin and cytokinin and Figs. 5–15 illustrate some of the interesting features to which further allusion is made below. Since in tobacco tissue culture the external phloem and most of the cambium presumably is removed during excision or destroyed in the sterilisation procedure, it is instructive to examine the origin of the callus produced in the subsequent cultures.

Origin of Callus

1. *Phloem parenchyma.* Phloem, if present, commonly gives rise to a thin layer of new growth as a result of a generalised proliferation of its parenchyma. In tobacco explants this type of growth is rare because, as previously pointed out, the phloem—and often also part of the cambium—is removed when the bark is peeled off in the explant technique. It was commonly observed, however, that the most superficial internal or intraxylary phloem bundles which were exposed by excision produced callus masses in which cambia frequently formed. (Fig. 5, ac, ng; Fig. 10, ca). Vegetative buds often formed immediately after proliferation of some callus from these phloem bundles, often without callus formation having occurred in other parts of the explant tissue. Rarely were the unexposed internal phloem bundles themselves observed to produce callus. The internal phloem imparts a measure of heterogeneity to the tobacco explant and while this type of tissue grows readily, interpretation of the results must take into consideration the controlling influences exerted by these centres of potential growth.

2. *Vascular cambium.* Commonly, the cambium, in those parts where it is undamaged, continues to divide and produces fine, downy flocks or pustules of new growth (Fig. 6, 7), the cells of which are large and intermingle to form a more or less continuous surface layer. Finally, the whole surface becomes greenish-white. Scattered vascular bundles of xylem and phloem appear, especially in treatments containing 4 mg/l IAA with little or no kinetin. Cambia regularly form at the boundary of parent tissue and are responsible for the development of the parenchymatous callus.

3. *Xylem parenchyma.* In the majority of cases the cambium of the explant is destroyed and a growth similar to that described above for cambial parenchyma originates from the cells of the xylem rays. Frequently, all the callus formed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in explant growth originated from a single or a few xylem rays (Figs. 8–9). In localized regions parenchymal meristems develop, the cells of which seem to retain a higher proliferative capacity than do parenchyma cells in general. Their structure distinguishes them from the normal pith parenchyma: they are vacuolate with a thin parietal cytoplasm and small nuclei, but do not seem to elaborate starch or other storage products. Their degree of differentiation appears to be intermediate to that of common parenchyma and cells of the cambium, and this probably explains their tendency to divide.

4. *Pith parenchyma.* At a first glance it is strange that in most cases the pith itself does not proliferate. Jablonksi and Skoog (1954) failed to obtain cell division in excised tobacco pith even with high concentrations of IAA, such as 4 mg/1. It seems as though the parenchyma of the xylem rays requires less external stimulation to divide than those of the pith. Figures 6 and 7 also show one of the rare instances where the pith parenchyma in fact divided and produced a callus mass.

Cambial activity

With the proliferation of the xylem rays into a homogeneous parenchymal tissue, cambia become organised in one of two ways. They either form in more or less linear tangential rows giving rise to phloem centrifugally and xylem—in regular radial rows—centripetally (Figs. 9, 11, 12), or circular cambia develop producing isolated vascular nodules or islets (Fig. 7). Internal cambia are a result of the dedifferentiation and reorganisation of parenchymatous elements, but once formed they proliferate tissues as do regular cambia. Whether these secondarily formed cambia are diffuse or well-defined seems to depend on the density of the callus mass which, in turn, is influenced initially by the hormone concentrations applied and probably also by carbon sources, as well as other factors.

Invariably, a cambium-like meristem is formed immediately beneath the surface layers of the callus. The growth here is primarily an expansion growth. As a result of repeated divisions, tangential walls form in the superficial parenchyma followed later by cell walls which now appear to be laid down randomly, thus forming groups of cells the composing elements of which often

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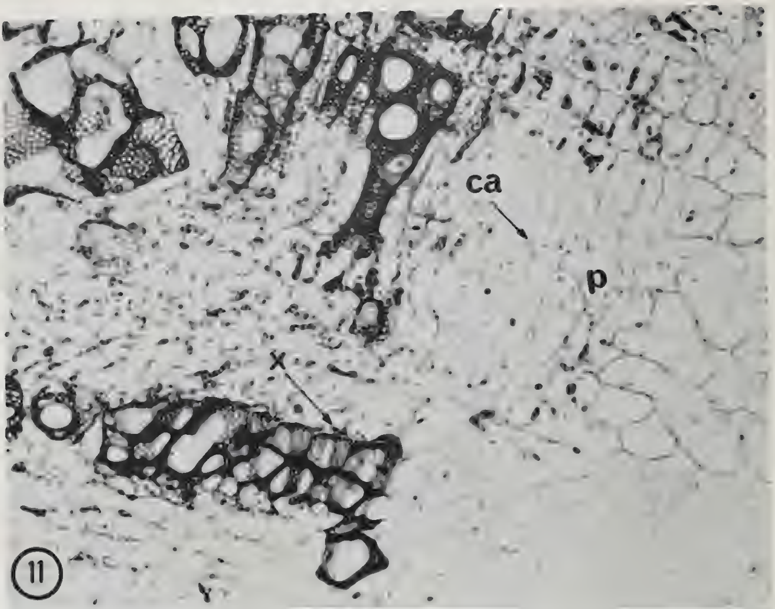
FIG. 10.

Transverse section of glutaraldehyde-fixed tissue piece, Fleming's triple stain. IAA:CK, 4·0:1·2 mg/1. Proliferation has occurred from the superficial intraxylary phloem bundles of the explant resulting in callus tissue in part of which a discrete cambium has formed regular, radial rows of cells. × 160.

c, callus; ca, cambium; ip, internal phloem; p, phloem; pp, pith parenchyma; px, parent xylem.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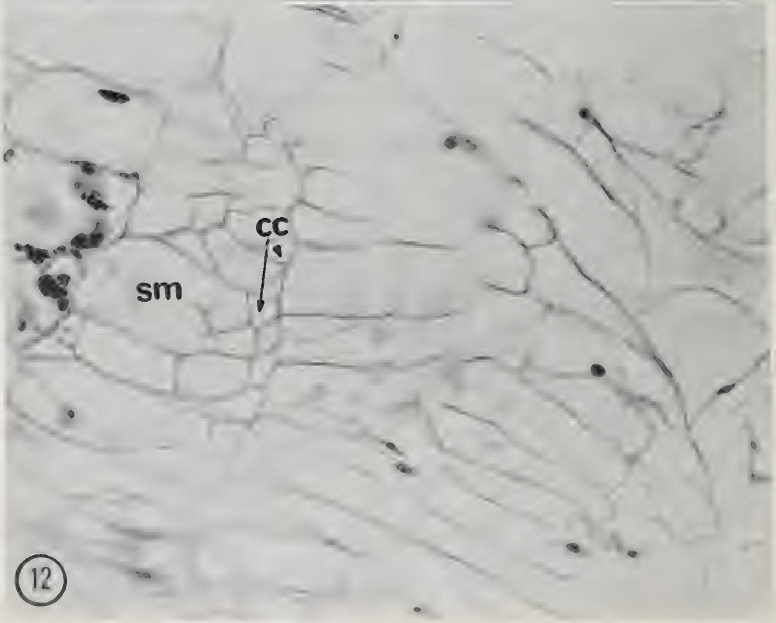
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exhibit radial arrangement. A layering results in which large lacunae are present in the outer zone.

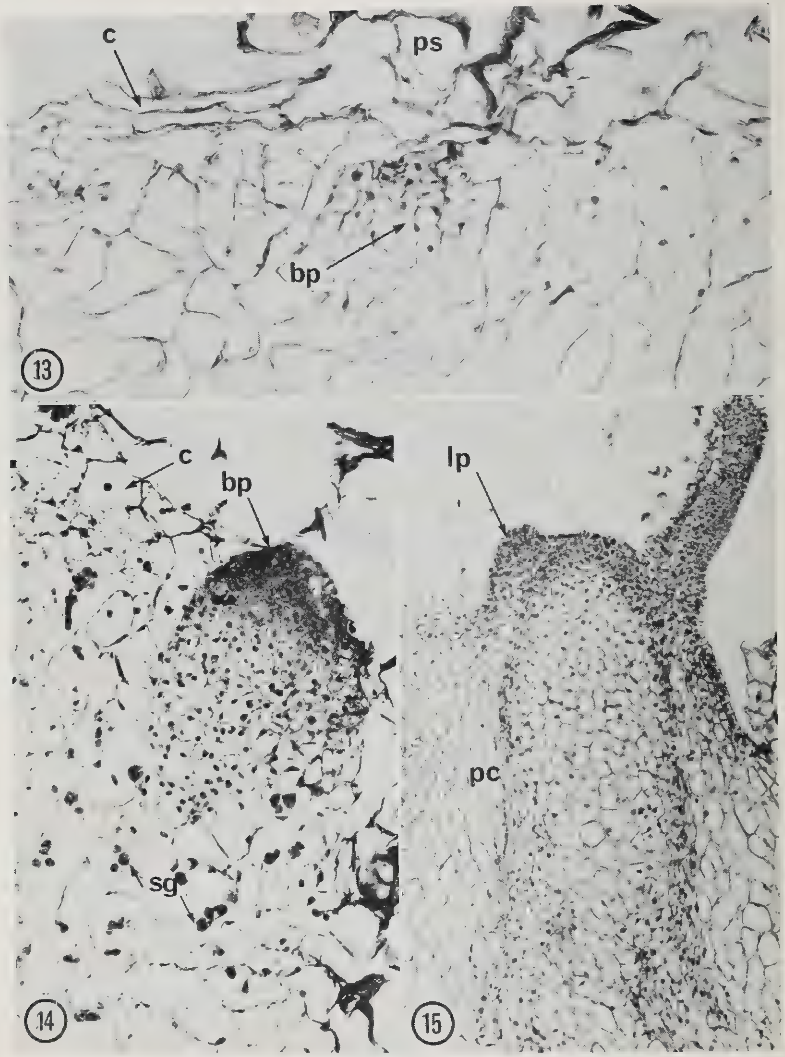
Spatial arrangement of new growth. Striking differences exist in structure between the new tissue growing into the nutrient medium and that developing into the air space above the agar. That into the agar consists of a fan-like proliferation forming a more compact, fairly regular meristematic tissue. The marginal cells of the proliferating tissue grow down into the agar and divide; the proximal division products are forced into close contact and form a compact tissue that remains actively dividing. There seem to be two ways in which this tissue keeps growing: (i) by pioneer action of the marginal cells; and (ii) by expansion caused by division of the cells of the adjoining tissue behind them. This explains the common observation of callus penetrating the agar surface, ultimately splitting it. Differentiation of cells, into tracheids for example, is exceedingly rare in this zone.

Above the agar, primary and secondary phases of growth may be recognised. The first phase corresponds to that in the agar except that the marginal cell columns are long and stretch out into the air space. The division products form a loose, irregularly constructed tissue of branches, often touching to form internal air spaces. Gautheret (1957) calls these more or less branched chains of cells "pseudothalli" and they are probably analogous to the "Wundhaare" of Magnus (1918). They remain free and do not intergrow. Through random divisions, however, they may lose their identity as all the air spaces (lacunae) become filled with cells. A full description of these pesudothalli is reported on in a subsequent paper. The secondary phase is that of differentiation and appears to depend on the concentration of IAA. Tracheids with scalariform or reticulate thickening and with walls which become lignified form in the pseudothalli.

Subcultured callus. The most common type of proliferation of subcultured callus is from the fundamental parenchyma. However, even such explants invariably contain vascular elements and vegetative growing points, the latter continuing development if the hormonal balance is conducive to either root or shoot formation or both. Growth is generally more uniform than is the case in stem explants, although vascular elements as well as vegetative meristems are formed in the same way.

FIGS. 11–12.

Histogenic differentiation in subcultured callus, Fleming's triple stain. IAA:CK, 4·0:1·2 mg/l. Fig. 11. Well-delineated cambium producing perforated tracheary elements and phloem. × 150. Fig. 12. Higher magnification of Fig. 10, showing detail of phloem. × 400. ca, cambium; cc, companion cell; p, phloem; sm, sieve tube member; x, xylem.



FIGS. 13–15.

Glutaraldehyde-fixed sections, haematoxylin-safranin stain. IAA:CK, 4·0:1·2 mg/l. ×160. Fig. 13. Very young stage of bud initiation indicated by group of meristematic cells constituting a bud primordium. Fig. 14. Developing bud primordium. Fig. 15. Longisection of a young bud with leaf primordium.

c, callus; bp, bud primordium; lp, leaf primordium; pc, procambial strand; ps, pseudothallial zone; sg, amyloplasts.

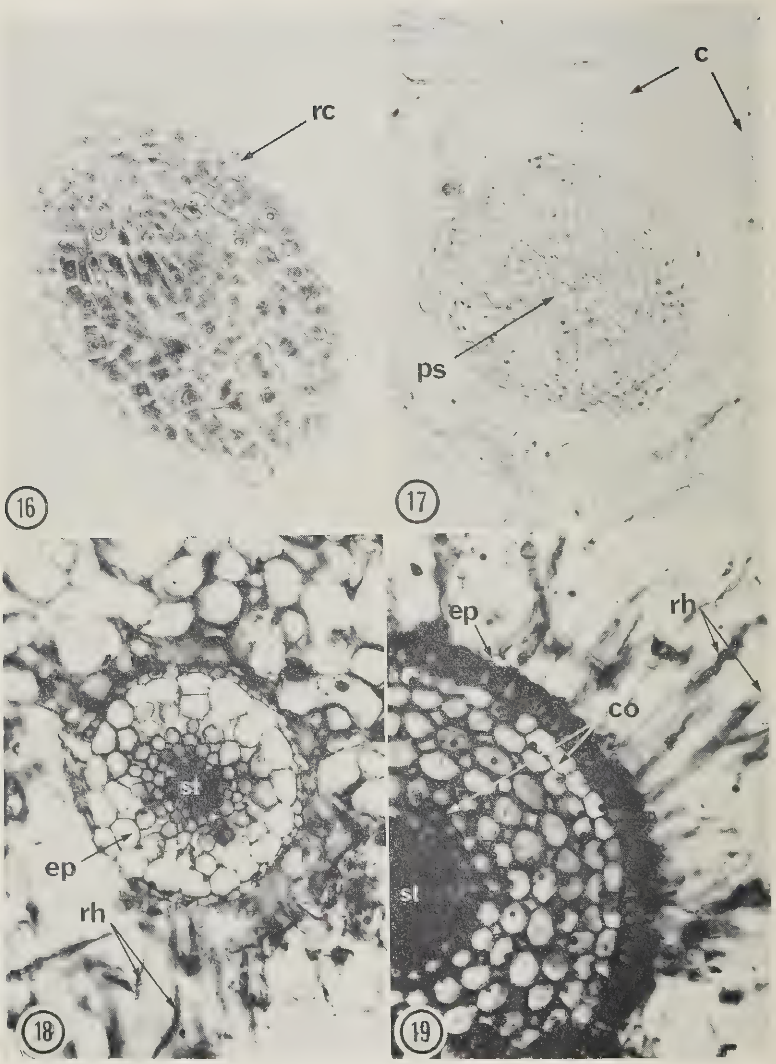
Histogenic and Organogenic differentiation. Most young culture colonies form well-defined cambia and it is from here that vascular structures are organised. These commence with the differentiation of islets of xylem around solitary or small groups of tracheids. Initially, cells surrounding these tracheids develop into rows of flattened cells that probably constitute a cambium, and ultimately result in the formation of cyclic nodules of vascular tissue, the composing elements of which often exhibit radial arrangement. High concentrations of auxin (4·0 mg/l IAA) produced large numbers of tracheids whereas high concentrations of cytokinin (1·2 mg/l kinetin) produced densely compacted xylem tissue.

In our experimental material the path of the vascular bundles could not be determined externally and properly oriented sections were extremely rare. However, as indicated in Figs. 11–12, phloem is a normal structure formed *in vitro* although it was not found associated with the islets of xylem.

Figures 13–15 show three successive stages in the organisation of a shoot. In roots a stele, cortex, and epidermal layer are soon formed immediately behind the meristematic apex deep in the callus (Figs. 16–19). The incidence of root formation in treatments of high auxin and low cytokinin was relatively frequent, ca. 65 per cent. However, a study of their ontogeny is not easy since they are initiated deep within the callus body and not in close association with any discernible structures. Young roots were often located in the callus and when retraced were found to disappear in the undifferentiated callus body. This is contrary to the observations of Steward, Mapes and Mears (1958) who found external roots associated with vascular strands in the callus.

Emergent roots frequently traversed up to 4 cm of callus tissue and when growing out through the callus the root cap behaved much as it would in soil. Root hairs (Fig. 18) were found to grow into the intercellular spaces of existing callus parenchyma. In the air space above the agar as well as in the agar itself root hairs were exceedingly numerous, each epidermal cell probably producing one. Near the apex, behind the root cap, the stele was found to be diarch but further back it is often triarch or polyarch. A frequent anomalous feature was the presence of "Siamese twins", two steles, reminiscent of *Selaginella*, enclosed within one epidermis. Three and even four steles were frequently observed, some with diarch and others with triarch steles. Eventually the steles branch out in dichotomy, each with its own root cap. Roots were often negatively geotropic.

As the cytokinin : auxin ratio increased the formation of roots, unorganised cellular proliferation, and the formation of shoots progressively increased in this sequence. Morphologically, like root meristems, shoot buds resulted from the dedifferentiation of the parenchymatous ground tissue formed by the cambium-like meristem at the surface (Fig. 13), followed by rapid division to



FIGS. 16–19.

Transverse sections through developing roots. IAA:CK, 4·0:0·08 mg/l. Fig. 16.Root. cap region. ×400. Fig. 17. Root, with polyarch stele, embedded in callus. ×160. Fig. 18 Root in callus. Note root hairs growing between cells of callus. ×160. Fig. 19. Tissue zones of root, showing numerous, long root hairs in callus. ×160. Figs. 18–19. Freehand sections of fresh tissue, aniline blue stain.

c, callus; co, cortex; ep, epidermis; ps, polyarch stele; rc, root cap; rh, root hair; st, stele.

form the meristematic bud primordia. Very early in the development of a bud an epidermis was formed from which enormous multicellular trichomes (up to 6·5 mm long) developed. Stomata often developed on the bases of trichomes on young buds. Not infrequently, shoots countered gravity and grew into the agar. Rather striking differences were observed in respect of shoot development when compared with the essential stages of organ initiation as reported by Steward *et al.* (1958). It must be kept in mind, however, that Steward *et al.* worked with liquid media. For example, specific areas with either root or shoot apices were not observed and seldom was there any vascular connection between the callus and even well-formed shoots.

CONCLUSION

Certain ontogenetic processes observed in cultured tissues are comparable to those in the intact plant. The organisation of a cambium between two tissues of different type is found both *in vitro* and in intact organs. However, the parenchymatous proliferation in cultured tissue is extremely rare in intact plants, and is limited, for example, to the formation of galls and tubers.

Tissue colonies appear to follow a regular and repeatable sequence of stages from their initiation to final completed development. The final form is governed to a large degree, if other factors such as light, temperature and carbon source are constant, by the ratios and concentrations of plant growth regulators supplied through the nutrient media.

REFERENCES

FEDER, N. and O'BRIEN, T. P., 1968. Plant microtechnique: some principles and new methods. *Amer. J. Bot.* **55**: 123–142.

GAUTHERET, R. J., 1957. Histogenesis in plant tissue cultures. *J. Nat. Cancer Inst.* **19**: 555–590.

JABLONSKI, J. R. and SKOOG, F., 1954. Cell enlargement and cell division in excised tobacco pith tissue. *Physiol. Plant.* **7**: 16–24.

JENSEN, W. A., 1962. *Botanical Histochemistry.* Freeman and Co., San Francisco.

JOHANSEN, D. A., 1940. Plant Microtechnique. McGraw-Hill Book Co.

KARSTENS, W. K. H., 1965. Phloem formation in explants and tissue culture, from an anatomical point of view. *Proc. Int. Conf. Plant Tissue Culture.* Eds. P. R. White and A. R. Grove.

MAGNUS, W., 1918. Wund-callus and bakterien-tumore. *Ber. deutsch. bot. Ges.* **36**: 20–29.

MURASHIGE, T. and SKOOG, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15**: 473–497.

SKOOG, F., 1957. Histogenesis in plant tissue cultures (discussion). *J. Nat. Cancer Inst.* **19**: 574–577.

VASIL, I. K. and HILDEBRANDT, A. C., 1966. Growth and chlorophyll production in plant callus tissues grown *in vitro. Planta* (Berl.) **68**: 69–82.

STEWARD, F. C., MAPES, M. O. and MEARS, K., 1958. Growth and organised development of cultured cells. II. Organisation in cultures grown from freely suspended cells. *Amer. J. Bot.* **45**: 705–708.